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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: :

Brake et al.

SERIAL NO.: 09/260,414 :

FILED: February 26, 1999 : Examiner: L. Lee

FOR: ATTENUATED LIVE NEOSPORA VACCINE : Art Unit: 1645

DECLARATION OF DAVID A. BRAKE, Ph.D.

Assistant Commissioner for Patents
Washington, D.C. 20231

I, DAVID A. BRAKE, Ph.D., declare and state as follows:

1. I am a named co-inventor of the above-identified application.

2. I have been employed in the Animal Health Biological Discovery Division of Pfizer Inc., which is the Assignee of the above-identified application, since 1995. From 1995 until 1999, I was a Senior Research Investigator in Animal Health Biological Discovery, Pfizer Inc. I have been a Manager in Animal Health Biological Discovery, Pfizer Inc., from 1999 to the present. A copy of my *Curriculum Vitae* is attached hereto as Exhibit A.

3. I have read and understand the complete specification and claims of the above-identified application. The above-identified application discloses methods for preparing cultures of cells of a strain derived from a pathogenic parent strain of the parasitic protozoan *Neospora caninum*, which cells exhibit attenuated pathogenicity compared to those of the parent strain but which are capable of triggering an immune response that protects a mammal against neosporosis when administered as a live vaccine. The above-identified application provides an example of a specific attenuated strain, *i.e.*, strain "NCTS-8", prepared from the pathogenic *Neospora caninum* parental strain NC-1, which is capable of triggering a protective immune response against neosporosis in an art-accepted animal model system when administered as a live vaccine.

4. I have also read and understand the outstanding Office Action dated September 15, 2000 ("Office Action"), which was received in the above-identified application.

5. I have also read and understand U.S. Patent No. 5,707,617 to Conrad et al., entitled "Bovine Neospora Isolates" (the "Conrad patent"), which is cited in the Office Action.

6. At my instruction, new mutagenized strains of *Neospora caninum* were prepared according to the guidance provided in the above-identified application and using the pathogenic *Neospora caninum* parent strain NC-2 as a novel source material. As demonstrated below, at least two of these new mutagenized strains, designated as "4D8" and "6H11", exhibit attenuated pathogenicity compared to parental strain NC-2, and are capable of triggering a protective immune response against neosporosis in an art-accepted animal model system when administered as a live vaccine. Strain 4D8 exhibits temperature selective growth, or "temperature sensitivity", whereas strain 6H11 does not.

7. The pathogenic NC-2 strain of *Neospora caninum* was originally isolated by a research group under the direction of Dr. David S. Lindsay, who is a named co-inventor on the above-identified application. Although murine neosporosis caused by strain NC-2 develops later and is somewhat less severe than that caused by strain NC-1, both strains are considered pathogenic. See Lindsay and Dubey, 1990, J. Parasitol. 76(3):410-413 (attached hereto as Exhibit B). Based at least on this work, NC-1 and NC-2 are distinguishable from each other as different strains of *Neospora caninum*.

8. Despite the biological differences between the NC-1 and NC-2 strains of *Neospora caninum*, the data presented below demonstrates that by following the guidance provided in the above-identified application a person of ordinary skill can prepare cultures of attenuated cells from strain NC-2, which cells are capable of triggering a protective immune response in mammals against neosporosis when used as a live vaccine. This data, along with that presented in the above-identified application as filed, demonstrates that such attenuated cells can be prepared from various different parental strains of *Neospora*. Accordingly, the disclosed invention is not limited to the use of only the NC-1 strain of *Neospora caninum*, but is generally applicable to other strains and species of *Neospora*. This conclusion is supported, for example, by the results presented in the Conrad patent (columns 15-17) demonstrating

the close antigenic similarity between tachyzoites of the two bovine *Neospora* isolates identified in the Conrad patent and those of *Neospora caninum*.

9. For the experiments described below, cells of *Neospora caninum* strain NC-2 were cultured and mutagenized essentially as described in the above-identified application. More specifically, cells of *Neospora caninum* strain NC-2 were cultured in HS68 human foreskin fibroblast cells at 37°C, and tachyzoites were mutagenized by exposure to N-methyl-N-nitro-N-nitrosoguanidine (0.5 μM) for 24 hr at 37°C. The mutagenized cells were selected for growth at 32°C for several months, after which tachyzoites were cloned by limited dilution. From three such mutagenesis procedures, a total of nineteen (19) positive clones were identified as potentially useful based on their ability to grow at 32°C.

10. To determine whether any of the novel strains prepared from NC-2 could trigger an immune response that would protect a mammal against neosporosis, an "adoptive-transfer" experiment was conducted as follows. The mouse model system used in these experiments is an art-accepted animal model for studying the development of neosporosis in mammals and for testing vaccine candidates. Female BALB/c mice (8-10 wk) were randomly assigned to different treatment groups (n = 7 or 8 mice per group), and immunized (s.c.) on days 0 and 21 with 1×10^6 tachyzoites of the particular strain to be tested. On or about day 35, three mice from each group were euthanized. Spleens were removed, and red blood cell-free single cell suspensions were prepared using standard methods. Recipient 4-5 wk old female SCID/bg (*i.e.*, immunodeficient) mice were randomly assigned to different treatment groups (n = 6 or 7 mice per group), and either received 10^7 spleen cells from BALB/c mice immunized with one of the novel NC-2 mutants or NCTS-8, or were sham-immunized. At day 36 (*i.e.*, one day post-cell transfer), all SCID/bg mice were challenged (s.c.) with 5×10^6 tachyzoites from virulent *Neospora* strain NC-1. Mice were monitored daily for survival. Survival curve data is presented in Graphs 1 and 2 (Exhibits C and D, attached hereto), which demonstrate that at least several novel strains, including strains 4D8 and 6H11, are capable of increasing the survival of the recipient SCID/bg mouse after NC-1 challenge, *i.e.*, they are protective. The graphs indicate that these survival curves are not significantly different from that involving adoptive transfer of spleen cells from mice immunized with NCTS-8. The graphs also show that at least one additional mutant strain, i.e., 4H3, does not provide significant protection over the sham-immunized control.

11. The data presented above demonstrates that at least 6 novel strains prepared from parental strain NC-2 can trigger protective immunity in mammals against neosporosis, similar to NCTS-8 prepared from NC-1. These 6 novel strains, which were designated as strains "2E4", "2F11", "3G4", "4D8", "6G7", and "6H11", were selected for further analysis using an art-accepted mouse neosporosis model system to determine whether they also exhibited reduced pathogenicity (*i.e.*, attenuation).

12. The 6 novel strains were tested for attenuation by determining their pathogenicity in comparison to *Neospora caninum* parental strains NC-1 and NC-2 and the attenuated strain NCTS-8. To demonstrate attenuation, tachyzoites (5×10^6/dose) from each of the 6 novel strains were isolated from host cell cultures grown at 32°C and inoculated (s.c.) into 4-6 wk old female SCID/bg mice, and survival rates of the mice were determined. Median days to death of the mice are presented below for each treatment in Table 1.

TABLE 1

Challenge Strain	Median Days to Death
4D8	26
NCTS-8	25.5
6H11	24
6G7	20
2E4	19
2F11	19
NC-2	19
NC-1	17.5
3G4	17

Based on the survival data, new strains 4D8 and 6H11 were comparable in attenuation to strain NCTS-8 exemplified in the above-identified application. Graph 3 (Exhibit E, attached hereto) presents the survival curve for each of the tested strains, and shows: (i) that the survival curves for strains NCTS-8 and 4D8 are each significantly different statistically from those of their respective parental strains ($p<0.05$); and (ii) that the survival curve for strain 6H11 is significantly different statistically from its parental strain NC-2 ($p<0.09$); both reflecting significantly increased survival (*i.e.*, lower pathogenicity).

13. The degree of attenuation was also determined by investigating the histopathological effect of these 6 novel strains in an art-accepted mouse neosporosis model system. Thus, 4-5 wk old BALB/c mice were immunosuppressed with methylprednisone acetate (MPA) at 7 days prior to challenge, and again on the day of challenge (day 0). The mice were then inoculated with the challenge strains (2×10^5 tachyzoites) on day 0. The mice were euthanized 56 days post-challenge, with the exception of the mice of the NC-1 inoculated group, which were euthanized on day 35 post-challenge, and their brain and lung tissues were examined for the presence of histopathological lesions. A summary of histopathologic data is presented in Table 2 below, in which a severity score for any given tissue alteration is as follows: 0 = no evidence of alteration (normal); 1 = minimal alteration; 2 = mild alteration; 3 = moderate alteration; and 4 = marked alteration.

TABLE 2

Challenge Strain	No. Survivors	Mean Brain Score	Mean Lung Score
4D8	5	0	0
NCTS-8	5	0	0
6H11	4	1	0
6G7	5	0.4	0.2
2E4	5	0.4	0.2
2F11	5	0.6	0.6
NC-2	5	1	0.2
NC-1	2	2	1.5
3G4	5	2.6	0.4
Control	4	0	0

As shown in Table 2, no tissue alterations were detected in animals challenged with strain 4D8, NCTS-8, or control (unchallenged) animals. Somewhat reduced histopathology was detected in animals challenged with strain 6H11 compared to its parental strain, NC-2. Animals challenged with other strains exhibited varying degrees of tissue alteration. Strain NC-1 produced the most severe tissue alteration, and also

resulted in the death of 3 out of 5 of the animals by day 35 post-challenge. Animals challenged with strain 3G4 were second in severity of tissue alteration, followed by animals challenged with strain NC-2 and 2F11. This data supports the conclusion that novel, protective strain 4D8 is an attenuated strain compared to its parent strain NC-2.

14. As a third indicator of attenuation, the ability of each of the 6 novel strains to grow at the temperature of the target mammalian species (*i.e.*, cattle) was determined. T25 flasks were seeded with 2×10^6 host green monkey kidney cells (strain MA104;), which were then inoculated the same day with 5×10^5 tachyzoites of the particular *Neospora* strain. For each strain, two flasks were maintained at 32°C and two flasks were maintained at 40°C. At five days post-inoculation, tachzoites were purified and counted. Graph 4 (Exhibit F, attached hereto), as expected, shows temperature selective growth in the strain NCTS-8 compared to its parental source strain NC-1. Whereas NC-1 has a higher rate of growth at 40°C compared to 32°C, strain NCTS-8 has a low rate of growth at both temperatures. In addition, graph 5 (Exhibit G, attached hereto) presents the data for the six novel strains prepared from parental strain NC-2 described above. Whereas strain NC-2 and five out of the six novel strains, including strain 6H11, all appear to have a higher rate of growth at 40°C compared to 32°C, strain 4D8 has a low rate of growth at both temperatures, indicating that this strain has an additional attribute indicative of attenuation.

15. By the criteria presented above (*i.e.*, attenuation and protective ability), Applicants have successfully used the teaching of their own disclosure to readily prepare and identify two more strains (*i.e.*, strains 4D8 and 6H11) from a pathogenic parent strain of *Neospora* that satisfy the claims of the above-identified application. The cells of strains 4D8 and 6H11 both exhibit attenuated pathogenicity compared to those of their parent strain, and both can trigger an immune response that protects a mammal against neosporosis when administered as a live vaccine. However, whereas strain 4D8 exhibits temperature sensitivity, strain 6H11 does not.

16. In view of the experimental results and remarks presented above, it is my professional opinion that a person of ordinary skill can use the ample guidance disclosed in the above-identified application to prepare other attenuated strains from any species or strain of *Neospora*, which attenuated strains would be capable of triggering protective immunity in mammals against neosporosis when administered as a live vaccine. It is also my professional opinion that such protective strains do not necessarily have to exhibit temperature selective growth or "temperature sensitivity". It

is also my professional opinion that, in view of the teaching of the above-identified application, the success of preparing such additional attenuated, protective strains does not rely on any specific method of mutagenesis, but can be achieved using any standard method of mutagenesis combined with appropriate selection protocols.

17. In my review of the Conrad patent, I observe that it is predominantly directed to a description of two new bovine *Neospora* isolates (BPA1 and BPA2) by characterizing their isolation, pathology, immunology in host animals, and nucleotide sequences encoding the nuclear small subunit ribosomal RNA. I further observe that the Conrad patent defines "Neospora antigen" as "an isolated Neospora tachyzoite, bradyzoite, or an isolated Neospora protein" (Conrad patent, column 1, lines 59-60). I further observe that the Conrad patent (Column 2, lines 15-19) purports to provide a "pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunologically effective amount of a bovine Neospora antigen", but in this regard it mentions only some unspecified "recombinantly produced bovine Neospora polypeptide." I further observe that the Conrad patent (column 11, lines 35-47) recites: "An attenuated Neospora vaccine can only be used in the absence of a risk of human infection should the milk or tissues of immunized animals be consumed." No other mention of the use of an attenuated *Neospora* appears anywhere in the Conrad patent. The Conrad patent provides no teaching as to how such an attenuated *Neospora* could be produced. In fact, the Conrad patent provides no reasonable expectation that preparation of an attenuated *Neospora* strain for use as a modified live vaccine would succeed. In addition, as seen in the above quote (¶ 17), Conrad teaches that the use of such vaccines is not preferred where the milk or tissues of the animal will be consumed. Accordingly, the Conrad patent teaches that such a vaccine would be practically useless in most circumstances.

18. Finally, I observe Example 5 of the Conrad patent (column 26, line 47 to column 28, line 6), which purports to demonstrate that three cows infected with a virulent *Neospora* isolate were subsequently protected against *Neospora*-induced abortion. In response to the assertions and conclusions made in Example 5 of the Conrad patent, I provide the following responses.

19. The testing described in Example 5 of the Conrad patent utterly fails to meet all basic standards of vaccine methodology. In the first instance, the small number of cows tested (n = 3) renders the observations statistically meaningless. Furthermore, the three cows were "immunized" with a virulent strain of bovine

Neospora that caused clinical disease. Such live virulent strains would never be utilized as vaccinal agents by a person of ordinary skill in the art.

20. Notwithstanding the assertion of protection in the Conrad patent (column 28, lines 3-5), no data is presented in the Conrad patent "to show that cattle can be protected against Neospora abortion by immunization with culture-derived tachyzoites of the BPA-1 Neospora isolate." Careful consideration of the data presented in the Conrad patent reveals the following. The virulent bovine isolate caused fetal infection in all three cows. All three cows were kept and rebred. According to the Conrad patent (column 26, lines 62-64), "All three experimental cows gave birth to seronegative, clinically normal calves (not all post mortem tissues examined to date)." The Conrad patent thus admits that not all of the data had been collected, and yet it concludes that the calves were "seronegative, clinically normal"! Such a conclusion is premature considering the incomplete nature of the data.

21. Based on my years of experience in the art of *Neospora* research, I am aware that the art currently recognizes that a single observation of seronegativity, by itself, is insufficient to support a determination as to whether a calf is infected with *Neospora*. The Conrad patent does not indicate that multiple blood samples were taken from the three cows. However, the art currently recognizes that at least three bleeds are required to establish any confidence in such serologic data.

22. I am also aware that the art currently requires that all three of: (i) serology; (ii) histopathology; and (iii) *Neospora*-specific PCR are to be analyzed to conclusively determine whether an animal has been infected with *Neospora*. For example, the art recognizes that a calf can be seronegative and yet give a PCR-positive test, thereby establishing that it is infected. In other words, it is known that a seronegative calf can still be infected with *Neospora*. Reliance on only negative serology can lead to the unfortunate situation where a calf that otherwise appears clinically normal is infected and capable of maintaining the infection in the herd. In view of this, it is my professional opinion that the serological results described in the Conrad patent fail to establish protection of the test animals.

23. The three cows described in Example 5 of the Conrad patent were rebred a second time, and then rechallenged with the virulent tachyzoites. Two of the three cows gave birth to live calves, which were purportedly "clinically normal and seronegative to Neospora antigens" (but see comments in ¶ 22 above). The third cow aborted 27 days post-inoculation. According to the Conrad patent (column 27, lines 6-

9), "mild lesions suggestive of Neospora infection were found, [although] Neospora infection, to date, has not been confirmed (formalin-fixed paraffin embedded tissues negative by histochemistry)." Regarding the lack of histochemical confirmation, I am aware that the art recognizes that formalin-fixed paraffin embedded tissue is not a preferred source for histochemical analysis. This is because formalin-fixed paraffin embedded tissue often gives false negative results as a result of the harsh, denaturing conditions involved in its preparation. The use of cryosectioned tissues is a more preferred method of conducting such histochemical analyses. In view of this, it is my professional opinion that the histochemical results described in the Conrad patent fail to establish protection of the test animals.

24. According to the Conrad patent, the third cow aborted its second pregnancy 27 days post-inoculation, resorbed its fetus in the third pregnancy, and aborted its fetus in its fourth pregnancy. Thus, at least one third (*i.e.*, one of three) of the cows described in Example 5 of the Conrad patent continued to experience symptoms of neosporosis after infection with the virulent bovine *Neospora* isolate. In view of this, it is my professional opinion that the breeding results described in the Conrad patent fail to establish protection of the test animals.

25. The Conrad patent (column 28, lines 3-6) concludes: "This is the first experiment to show that cattle can be protected against Neospora abortion by immunization with culture-derived tachyzoites of the BPA-1 Neospora isolate." I strongly disagree with this conclusion based on: (i) the lack of complete data (*i.e.*, not all post-mortem tissues had been examined to date); (ii) the lack of sufficient data (*i.e.*, the small sample size (n=3), the use of only serological testing in some instances, and the use of formalin-fixed paraffin embedded tissue in others); (iii) the occurrence of fetal infection or abortion in 3 out of 3 cows following first "immunization" with the culture derived tachyzoites; and (iv) the continuing occurrence of failed pregnancies in at least one of three cows tested.

26. Based on the above review, it is my professional opinion that the Conrad patent fails to establish that cattle can be protected against *Neospora*-induced abortion by immunization with culture-derived tachyzoites of the BPA-1 *Neospora* isolate.

27. Based on the above analysis, it is also my professional opinion that the Conrad patent would not have taught or suggested cultures of cells of a strain derived from a pathogenic parent strain of the parasitic protozoan *Neospora caninum*, which cells exhibit attenuated pathogenicity compared to those of the parent strain but which

are capable of triggering an immune response that protects a mammal against neosporosis when administered as a live vaccine, methods of preparing such cultures, or vaccines based on such cultures.

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: November 21, 2000

David A. Brake
Pfizer Central Research
Groton, CT.

Attachments

Exhibit A: *Curriculum Vitae* of David A. Brake, Ph.D.

Exhibit B: Article by Lindsay and Dubey, 1990, J. Parasitol. 76(3):410-413.

Exhibit C: Graph 1 – Evaluation of protective ability of several *N. caninum* mutants

Exhibit D: Graph 2 – Evaluation of protective ability of several *N. caninum* mutants

Exhibit E: Graph 3 – Comparison of pathogenicity of *N. caninum* mutants

Exhibit F: Graph 4 – In vitro growth characteristics of NCTS-8 and NC-1

Exhibit G: Graph 5 – In vitro growth characteristics of NC-2 mutants

DAVID ALAN BRAKE
Curriculum Vitae

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Personal Data:
Born: September 8, 1960
Married, with 2 children (Elliot and Lee)

Education:

Hahnemann University 1982-1987
Ph.D. Microbiology/Immunology
Thesis: Cell-Mediated Mechanisms of Immunity to *Plasmodium chabaudi adami*

Muhlenberg College 1978-1982
B.S. Biology

Research and Work Experience:

Pfizer, Inc.

1999-present. Manager. Animal Health Biological Discovery. Responsibilities include project manager for livestock discovery projects (11 projects), providing leadership for the Biological Evaluation group and facilitating the implementation of Animal Health Central Research operating and strategic plans. Currently supervise a staff of 15 scientists.

1995-1999. Senior Research Investigator. Animal Health Biological Discovery. Duties include project team coordinator for Abortion Prevention Project (4 individual projects) and manager for all internal and external parasite vaccine opportunities. Directly supervise 1 assistant scientist.

SmithKline Beecham Animal Health

1993-1995. Project Manager, Dept. of Immunology.
Established and directed clinical immunology assay group to support all viral and bacterial vaccine livestock and companion animal projects. Project leader for internal parasite project and all external parasite initiatives. Directly supervised 5 scientists (1 Ph.D., 2 M.S., 2 B.S.) with responsibility for $500K operating budget.

1992-1993. Senior Investigator, Dept. of Molecular Biology.

Directed Immunology laboratory responsible for support of all viral and parasite vaccine projects, including major avian vaccine project. Directly supervised 2 scientists (1 post-doctorate, 1 M.S.) and summer hires.

1990-1992. Associate Senior Investigator, Dept. of Molecular Biology. Provided immunological expertise to assigned animal vaccine projects.

SmithKline Beecham Pharmaceuticals

1987-1990. PostDoctoral Fellow, Dept. of Molecular Genetics. Basic molecular and cell biology research on HIV gene regulation.

SmithKline Clinical Laboratories

1984-1987. Medical Laboratory Technologist, Clinical Microbiology. Part-time position working in microbiology lab at Hahnemann University Hospital.

Areas of Research Interest and Technical Expertise:

Research interests: bacterial, parasite and viral immunology with emphasis on vaccine design and evaluation using in vitro assays and experimental animal models.

Technical expertise: *multifaceted immunological techniques* (CMI assays, T cell lines and clones, Mab production, SDS-PAGE/Westerns, cytokine and antibody ELISAs for livestock and companion animal species; *molecular biology techniques (* cDNA and genomic cloning/subcloning; Southern and Northern blot analysis; RT-PCR, DNA sequencing; nucleic acid/protein interactions; in-vitro transcription/translation*); bacterial and mammalian recombinant protein expression.*

Honors and Professional Society Memberships:

Sigma X
Who's Who Among Students in American Universities and Colleges
Dean's Award, Hahnemann University, 1986
Panelist/Reviewer, NIH SBRI Program, 1994-1996
Ad Hoc Reviewer, Infection and Immunity; Molecular and Cellular Probes
American Society of Microbiology
American Association of Veterinary Immunologists
American Association of Veterinary Parasitologists
New England Association of Parasitology

Publications:

1. D.S. Lindsay, D.M. Ritter and D.A. Brake. 2000. Oocyst excretion in dogs fed mouse brains containing tissue cysts of a cloned line of *Neospora caninum*. J. Parasitol. Submitted.
2. Coyne, C.P. and Brake, D.A. 2000. Characterization of *Haemonchus contortus*-derived cell populations propagated in vitro in a tissue culture environment: potential immunoprotective properties in sheep. Biochemistry and Cell Biology. Submitted.
3. Dreier, K.J., Stewart, L.W., Kerlin, R.L., Ritter, D.M. and Brake, D.A. 1999. Phenotypic characterization of a *Neospora caninum* temperature-sensitive strain in normal and immunodeficient mice. Intl. J. Parasitol. 29:1627-1634.
4. Lindsay, D.S., Lentz, S.D., Blagburn, B.L. and Brake, D.A. 1999. Characterization of temperature-sensitive strains of *Neospora caninum* in mice. J. Parasitol. 85:64-67.
5. Brake, D.A., Fedor, C.H., Werner, B.W., Taylor R.L., and Clare, R.A. 1997. Development of a natural immune model to *Eimeria tenella* in different major histocompatibility complex hosts and initial immune response characterization to tissue culture-derived *E. tenella* antigens. Infect.Immun.65:1204-1210.
6. Brake, D.A., Strang, G., Lineberger, J.E., Fedor, C.H., Clare, R.A., Banas, T. A., and Miller, T. 1997. Characterization of a tissue culture-derived vaccine which affords partial protection against avian coccidiosis. Poultry Sci..76:974-983.
7. Brake, D.A. and Debouck, C. 1991. The HIV-1 Tat transactivator contains an arginine-glycine-aspartyl (RGD) cell adhesion site. In Advances in Molecular Biology and Targeted Treatment for AIDS (Kumar, A., ed.). 145-150.
8. Brake, D.A., Debouck, C., and Biesecker, G. 1990. Identification of an Arg-Gly-Asp (RGD) Cell Adhesion Site in Human Immunodeficiency Virus Type I Transactivating Protein, tat. J. Cell Biol. 111:1275-1281.
9. Brake, D.A., Goudsmit, J., Krone, W.J.A., Schammel, P., Appleby, N., Meloen, R.H. and Debouck, C. 1990. Epitope mapping and functional analysis of tat protein from human immunodeficiency virus by monoclonal antibodies. J. Virol 64:962-965.
10. Goldring, J.P., Brake, D.A., Cavacini, L.A., Long, C.A. and Weidanz, W.P. 1989. Cloned T cells provide help for malaria-specific polyclonal antibody responses. Eur. J. Immunol. 19:559-561.
11. Weidanz, W.P., Brake, D.A., Cavacini, L.A. and Long, C.A. 1988. The protective role of T cells in immunity to malaria. In Host Defenses and Immunomodulation to Intracellular Pathogens. (Eds. Eisenstein, Bullock, Hanna) p. 99-111.
12. Brake, D.A., Burns, J.M., Weidanz, W.P., Vaidya, A.B. and Long, C.A. 1988. Adoptive protection in nude mice against a murine malarial parasite using a cloned T cell line. In Vaccines 88. (Eds. Ginsberg, Brown, Lerner, Chanock) p. 85-88.
13. Brake, D.A., Long, C.A. and Weidanz, W.P. 1988. Adoptive protection against *Plasmodium chabaudi adami* malaria in athymic nude mice by a cloned T cell line. J. Immunol. 140:1989-1993.
14. Brake, D.A., Weidanz, W.P. and Long. C.A. 1986. Antigen-specific interleukin 2-propagated T lymphocytes confer resistance to a murine malarial parasite, *Plasmodium chabaudi adami*. J. Immunol. 137:347-352.

Invited Speaker

1. Brake, D.A. 1999. Vaccine Approaches for Control of Bovine Neosporosis. Dept. of Veterinary and Animal Sciences. Univ. Massachusetts-Amherst.
2. Brake, D.A. 1999. Animal Health Vaccines: New Definitions for the New Millennium. Connecticut United for Research Excellence (CURE) Biotechnology Seminar Series.
3. Brake, D.A. 1998. Parasite Vaccines: Insights into Building New Biological Weapon Systems for Testing at the Host-Pathogen Front, 43rd Annual Meeting of the American Association Veterinary Parasitology.
4. Brake, D.A.. 1992. Biotechnology and new considerations for vaccine development for coccidiosis. 27th National Meeting on Poultry Health and Processing.

Select Abstracts and Presentations

1. Lindsay, D.S., Ritter, D. and Brake, D.A.. 2000. Oocyst production in dogs fed tissue cysts of a cloned bovine strain of *Neospora caninum*. Abstract 121. American Society of Parasitologists and The Society of Protozoologists.
2. Dreier, K.D., Ritter, D.M. and Brake, D.A. 1999. Adoptive Protection Against Acute Neosporosis in Mice. 44th Annual Meeting of the American Association Veterinary Parasitology.
3. Dreier, K.D., Ritter, D.M. and Brake, D.A. 1999. Adoptive Protection Against Acute Neosporosis in Mice. 3rd Wood's Hole Immunoparasitology Meeting.
4. Brake, D.A., Strang, G. and Miller, T. 1997. Local immune responses to extracellular and intracellular *Eimeria tenella* antigens in naïve and immune chickens following infection. VIIth International Coccidiosis Conference
2. Baarsch, M.J., Warren, L., Brake, D.A and Campos, M.C. 1995. *Actinobacillus pleuropneumoniae*: measurement of the acute phase response following serotype 5 challenge. 4th International Veterinary Immunology Conference.
3. Dreier, K.J., Terwee, J. and Brake, D.A. 1995. Measurement of α-1 acid glycoprotein levels during feline viral and bacterial infection and correlation to clinical disease. Seventy-Sixth Conference of Research Workers in Animal Diseases.
4. T.D.Yule, M. Roth, K. Dreier, D. Brake, T. Johnson, S.W. May and M. Sabara. 1994. Panleukopenia infection in 14 week old SPF cats: correlation of peripheral blood differential cell counts with flow cytometric analysis and alpha-1 acid glycoprotein levels. Seventy-Fifth Conference of Research Workers in Animal Diseases.
5. K.M. Kokjohn, K.J. Dreier, K.C. Shain, J. TerWee and D. Brake. 1994. Detection of IL-6 in feline plasma samples using a commercial ELISA kit. Seventy-Fifth Conference of Research Workers in Animal Diseases.

Patent Applications

1. Krishnan, B.R. , Coleman, R.C., Yoder, C.C., Durtschi, B.A. and Brake, D.A. (assignee Pfizer, Inc.). Polynucleotide molecules encoding *Neospora* proteins (filed March 26, 1998), PC9943.
2. Brake, D.A. and Campos, M.C. (assignee Pfizer, Inc.). *Neospora* vaccine (filed August 26, 1997), EP 98306431.
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INFECTIONS IN MICE WITH TACHYZOITES AND BRADYZOITES OF *NEOSPORA CANINUM* (PROTOZOA: APICOMPLEXA)

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ABSTRACT: Tachyzoites of 2 isolates of *Neospora caninum* (NC-1 and NC-2) were inoculated subcutaneously (s.c.), intraperitoneally (i.p.), or orally into mice to compare the effects of route of inoculation on pathogenicity. Mice developed more severe disease, and disease occurred sooner when inoculated with the NC-1 isolate compared to the NC-2 isolate. Deaths occurred earlier in mice inoculated i.p. with either isolate. Mice inoculated orally or s.c. with tachyzoites responded similarly to infection. Tissue cysts of the NC-2 isolate produced infections in mice following oral or s.c. inoculation. Lesions seen in mice inoculated with tachyzoites or bradyzoites were primarily acute pneumonia, myositis, encephalitis, ganglioradiculoneuritis, and pancreatitis. In vitro studies demonstrated that tachyzoites of both isolates were killed by incubation in pepsin-HCl solution but not 1% trypsin solution. Bradyzoites of the NC-2 isolate were able to withstand treatment with pepsin-HCl solution.

Neospora caninum is an apicomplexan parasite that causes severe disease in dogs (Cummings et al., 1988; Dubey and Beattie, 1988; Dubey, Carpenter et al., 1988; Dubey, Hattel et al., 1988; Dubey and Lindsay, 1989a; Hay et al., 1990). It was first described and isolated from naturally infected dogs in 1988 (Dubey, Carpenter et al., 1988; Dubey, Hattel et al., 1988). Until that time it had been confused with the structurally similar parasite *Toxoplasma gondii* (Dubey and Beattie, 1988; Dubey, Carpenter et al., 1988). Canine neosporosis is characterized by encephalitis, polyradiculoneuritis, polymyositis, and ascending paralysis.

Many aspects of the life cycle and sources of infection are not known. Tachyzoites, rapidly dividing stages, are found in many types of cells in many tissues. Tissue cysts contain bradyzoites, slowly multiplying stages, and are found only in the central nervous system (CNS). The parasite can be transmitted transplacentally (Dubey, Hattel et al., 1988; Dubey and Lindsay, 1989a, 1989b). Tachyzoites and tissue cysts develop in extraintestinal tissues of animals. These similarities to *T. gondii* indicate that other stages of a coccidian life cycle might develop in the intestines of a yet unknown definitive host.

A cat fed tissues containing *N. caninum* cysts and tachyzoites became infected (Dubey and Lindsay, 1989b). Whether the infection occurred via the buccal mucosa or via stages surviving passage through the stomach is not known, nor is the stage responsible for initiating the infection. However, this does demonstrate that stages of *N. caninum* are infective following ingestion.

Experimental infections in methylprednisolone acetate (MPA)-treated mice have provided a laboratory model to study aspects of the transmission, pathogenesis, and chemotherapy of *N. caninum* infections (Lindsay and Dubey, 1989b). The mouse model was developed using subcutaneous (s.c.) inoculation of tachyzoites. Detailed experiments on the infectivity of *N. caninum* tissue cysts and effects of route of inoculation of tachyzoites on infectivity and pathogenesis have not been reported.

In the present study, we examined the effects of oral, s.c., and intraperitoneal (i.p.) inoculation of tachyzoites of 2 different isolates of *N. caninum*, and oral and s.c. inoculation of bradyzoites of 1 isolate in MPA-treated mice. We also conducted in vitro studies to determine the resistance of tachyzoites and bradyzoites to the digestive enzymes, pepsin and trypsin.

MATERIALS AND METHODS

Parasites and preparation of inocula

Tachyzoites of the NC-1 (Dubey, Hattel et al., 1988) and NC-2 (Hay et al., 1990) isolates of *N. caninum* were isolated originally from naturally infected puppies. Both isolates are maintained by continuous passage in bovine monocyte (BM) cell cultures (Lindsay and Dubey, 1989a) that are grown in culture medium consisting of RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml dihydrostreptomycin, 50 U/ml penicillin G (GIBCO, Grand Island, New York), and 5×10^{-2} 2-mercaptoethanol (Sigma Chemical Company, St. Louis, Missouri). To obtain tachyzoites from host cells, monolayers were scraped off the plastic growth surface, which ruptures most in-

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EXHIBIT B

fected BM cells, and the culture medium was then filtered through a sterile 3-μm filter. Tachyzoites were counted in a hemacytometer, and the volume of solution was adjusted with Hanks' balanced salt solution (HBSS) so that 1 ml contained 2×10^5 tachyzoites for s.c. inoculations and 0.5 ml contained 2×10^5 tachyzoites for oral or i.p. inoculations.

Bradyzoites were obtained from tissue cysts in the brains of 2 mice inoculated 6 mo previously with the NC-2 isolate. One mouse had mild posterior paralysis, and tissue cysts were identified in a brain smear. The other mouse had no clinical sign, and tissue cysts were not seen in a brain smear. The brains of both mice were triturated together in HBSS using a mortar and pestle, and a portion of the mixture was digested in pepsin-HCl solution (pH 0.8) (Dubey and Beattie, 1988) for 10 min. The digested mixture was washed 3 times in cell culture medium by centrifugation prior to use. Portions of undigested brain mixture were used for oral inoculations, and portions of the digested mixture were used for oral and s.c. inoculations. The number of cysts or bradyzoites that each mouse received was not determined.

Inoculation and examination of mice

Female Swiss white mice (20–25 g) were used for inoculations. The number of mice inoculated, the isolate used for inoculation, and the route of inoculation are given in Table I. All mice inoculated with *N. caninum* were given intramuscular injections of 4 mg MPA (Med-Tech Inc., Elwood, Kansas) on days −7, 0, and 7 postinoculation (PI) of parasites to facilitate detection of *N. caninum* stages (Lindsay and Dubey, 1989b). Control groups consisted of mice that were given only MPA and mice that were not given MPA or tachyzoites.

Tissues were collected for histological examination from mice that were killed while moribund, mice judged to be recently dead, and all mice that were killed when the study was terminated 28 days PI. Portions of brain, spinal column, tongue, lung, heart, diaphragm, thigh muscle, pancreas, adrenal, kidneys, and liver were fixed in neutral buffered 10% formalin and processed for light microscopic examination after staining with hematoxylin and eosin. Fresh smears were made from the lungs of mice that died and were examined immediately for tachyzoites with light microscopy.

In vitro assay for effects of pepsin-HCl and trypsin solutions on parasites

Tachyzoites of *T. gondii* survive in trypsin but not in pepsin solutions, whereas bradyzoites survive in both solutions (Jacobs et al., 1960). To determine the effects of these digestive enzymes on *N. caninum*, monolayers of infected BM cells containing tachyzoites of either the NC-1 isolate or the NC-2 isolate were scraped from the plastic growth surface, pelleted by centrifugation, and exposed to pepsin-HCl solution (pH 0.8) or 1% (w/v) trypsin in HBSS (pH 5.5) for 30 min. The samples were then washed by centrifugation in cell culture medium and inoculated onto monolayers of uninfected BM cells. Portions of the undigested and pepsin-HCl-digested brain mixture were processed and inoculated similarly. Cell cultures were then examined for 30 days for growth of *N. caninum* (Lindsay and Dubey, 1989a).

TABLE I. Protocol for inoculation of mice with tachyzoites (T) or bradyzoites (B) of *Neospora caninum* and results of inoculations.

Group	Isolate	Mode*	Digested	MPA†	No. infected/ no. inoculated
1	NA‡	NA	NA	None	0/5
2	NA	NA	NA	Yes	0/10
3	NC-1	s.c.-T	No	Yes	10/10
4	NC-1	i.p.-T	No	Yes	10/10
5	NC-1	O-T	No	Yes	10/10
6	NC-1	O-T	No	Yes	10/10
7	NC-2	s.c.-T	No	Yes	5/5
8	NC-2	i.p.-T	No	Yes	10/10
9	NC-2	O-T	No	Yes	4/5
10	NC-2	s.c.-B	Yes	Yes	10/10
11	NC-2	O-B	Yes	Yes	7/10
12	NC-2	O-B	No	Yes	1/5

* s.c., subcutaneous inoculation; i.p., intraperitoneal inoculation; O, oral inoculation.
† Methylprednisolone acetate.
‡ Not applicable.

RESULTS

In vivo experiments

Mice inoculated s.c. with tachyzoites of either isolate developed acute neosporosis, and all were infected when examined at necropsy (Table I). However, differences in the responses of mice to s.c. inoculation of the 2 strains were noted. All 10 mice inoculated s.c. with the NC-1 isolate (group 3) developed pneumonia and died or were killed when moribund 7–11 days PI, whereas 3 of 5 mice inoculated s.c. with the NC-2 isolate (group 7) died 15–17 days PI. One of the remaining mice in group 7 had a paralyzed right hind leg, and the other mouse was normal when killed 28 days PI. Lesions seen in the tissues of mice in group 3 consisted mainly of acute pneumonia. Encephalitis, pneumonia, myositis, myocarditis, and pancreatitis were seen in the tissues of the 3 mice in group 7 that died 15–17 days PI. The 2 surviving mice in group 7 had lesions in the brain and spinal cord that consisted of vasculitis, gliosis, and mineralization. Ganglioradiculoneuritis also was seen in sections of the spinal columns of these mice.

Mice inoculated i.p. with tachyzoites of either isolate all became infected (Table I). Five of 10 mice inoculated i.p. with the NC-1 isolate (group 4) died 4 days PI and the remaining 5 were moribund and killed on this day. Mice inoculated i.p. with the NC-2 isolate (group 8) reacted similarly to infection; 1 died 6 days PI and 2 were killed when moribund on this day. Two group 8 mice died 7 days PI and the remaining 5 were

killed on this day. Minimal amounts (0.3–0.8 ml) of yellowish ascites fluid were present in the abdominal cavities of mice in groups 4 and 8. Microscopic examination of the ascites fluid revealed many inflammatory cells but few tachyzoites. Lesions were confined to the visceral tissues. The most severe microscopic lesions were seen in the pancreas of infected mice and consisted of diffuse necrosis of acinar cells (mainly at the margins of pancreatic lobes) and infiltration of pancreatic connective tissue by inflammatory cells. Minimal lesions were seen also in the livers, adrenal glands, and abdominal diaphragm of i.p.-inoculated mice and consisted of small areas of necrosis or myositis.

Mice inoculated orally with tachyzoites of either *N. caninum* isolate became infected (Table I) and isolate-related differences in responses were observed. Fourteen of 20 mice inoculated orally with the NC-1 isolate (groups 5 and 6) died of acute pneumonia 7–12 days PI, 2 died from encephalitis 26 days PI, 1 died from encephalitis 28 days PI, and 3 were killed 28 days PI at the end of the study. One of the 3 surviving mice had a head tilt to the right indicating encephalitis; the other 2 appeared normal. None of 5 mice inoculated orally with the NC-2 isolate developed clinical neosporosis or died. However, 4 of these mice had lesions in the brain consistent with neosporosis, and tachyzoites were seen in 2 of these 4 mice. Microscopic lesions seen in orally inoculated mice were similar to s.c.-inoculated mice examined at the same day PI.

Subcutaneous inoculation of pepsin-digested brain mixture of the NC-2 isolate (group 10) produced infections in 10 of 10 mice (Table I). Four mice died 14–16 days PI, and the remaining 6 mice were killed 28 days PI. Three of the 6 mice had clinical signs; 2 had head tilts and 1 had hind leg paralysis. Microscopic lesions were present in the 6 mice and were similar to those seen in mice inoculated with tachyzoites.

Oral inoculation of mice with brain mixture of the NC-2 isolate produced infections in the mice (Table I). Oral inoculation of undigested brain material (group 12) produced infection in only 1 of 5 mice. The infected mouse died 14 days PI and had mild myositis and severe pneumonia. However, fungal hyphae were also seen in the lungs of this mouse, and its death can not be attributed to *N. caninum*-induced pneumonia. Oral inoculation of mice with pepsin-digested brain containing cysts produced infections in 7 of 10 mice (group 11). One of the 10 mice died 17 days PI; the other 9 were killed 28 days PI. Pneumonia and encephalitis were present in the mouse that died. None of the 9 mice had clinical signs when killed. The 6 infected mice killed 28 days PI had lesions of encephalitis and ganglioradiculoneuritis. Tachyzoites were seen in the brains of 2 of these 6 mice. Lesions were similar to those of other *N. caninum*-infected mice.

Neospora caninum tissue cysts were not observed in any mouse in the present study. Mice that did not receive tachyzoites or MPA (group 1) and mice that received only MPA (group 2) remained healthy during the study. Mice in group 2 usually had small or inapparent thymuses and mesenteric lymph nodes. No microscopic lesion was seen in the tissues of mice in groups 1 or 2.

In vitro experiments

Bovine monocyte cell cultures that were inoculated with undigested and digested brain material containing cysts of the NC-2 isolate became infected. This indicates that cysts were present in the undigested inoculum and that bradyzoites can survive digestion in pepsin-HCl solution. Exposure of tachyzoites of both isolates to pepsin-HCl solution was lethal; none of the BM cell cultures became infected. Exposure of tachyzoites of both isolates to 1% trypsin solution was nonlethal; both isolates were infectious for BM cells after incubation.

DISCUSSION

The pathogenicity of the NC-1 isolate has been characterized previously in MPA-treated mice following s.c. inoculation (Lindsay and Dubey, 1989b). The present study provides information on oral and i.p.-induced infections. It demonstrated that mice are susceptible to oral inoculation with tachyzoites and that the infections produced were similar to those produced by s.c. inoculation. Intraperitoneal inoculation of tachyzoites was found to differ from both s.c. and oral routes of inoculation in that mice died earlier in the infection, before pneumonia, myositis, or encephalitis developed.

The NC-1 isolate appears to be more pathogenic for mice than does the NC-2 isolate. Disease developed later, was less severe, and more mice survived to the end of the study when inoculated with tachyzoites of the NC-2 isolate.

Bradyzoites from tissue cysts of the NC-2 isolate were shown to be infective following oral or s.c. inoculation. The resulting infections were

similar to tachyzoite-induced infections. Not all mice inoculated orally with tissue cysts became infected. The low infectivity of undigested brain mixture is probably related to the low numbers and uneven distribution of tissue cysts in the inoculum.

Results of the in vitro studies to characterize the resistance of tachyzoites to pepsin-HCl and trypsin solutions indicated that pepsin-HCl was lethal, whereas trypsin solution was not. This is also true for tachyzoites of *T. gondii* (Jacobs et al., 1960). The inability of *N. caninum* tachyzoites to withstand pepsin-HCl solution also indicates that cysts are not present in the cell cultures because *N. caninum* bradyzoites were not killed by incubation in pepsin-HCl solution. The tissue cyst wall of *T. gondii* is destroyed soon after incubation in pepsin solution, and the released bradyzoites can survive for several hours (Jacobs et al., 1960). We did not examine the effect of pepsin solution on the tissue cyst wall of *N. caninum*.

The most puzzling aspect of the present study is the infectivity of orally inoculated tachyzoites. Results of in vitro studies showed that tachyzoites were killed by pepsin-HCl solution, indicating that they should not have survived passage through the stomach. There are 2 possible explanations for the observed results: (1) tachyzoites are infective by oral inoculation and can survive passage through the stomach, and (2) during inoculation the tachyzoites can penetrate the mucosa of the oral cavity/esophagus, bypass the stomach, and produce infection. The latter route of infection might be aided by any trauma to the mucosa resulting from inoculation.

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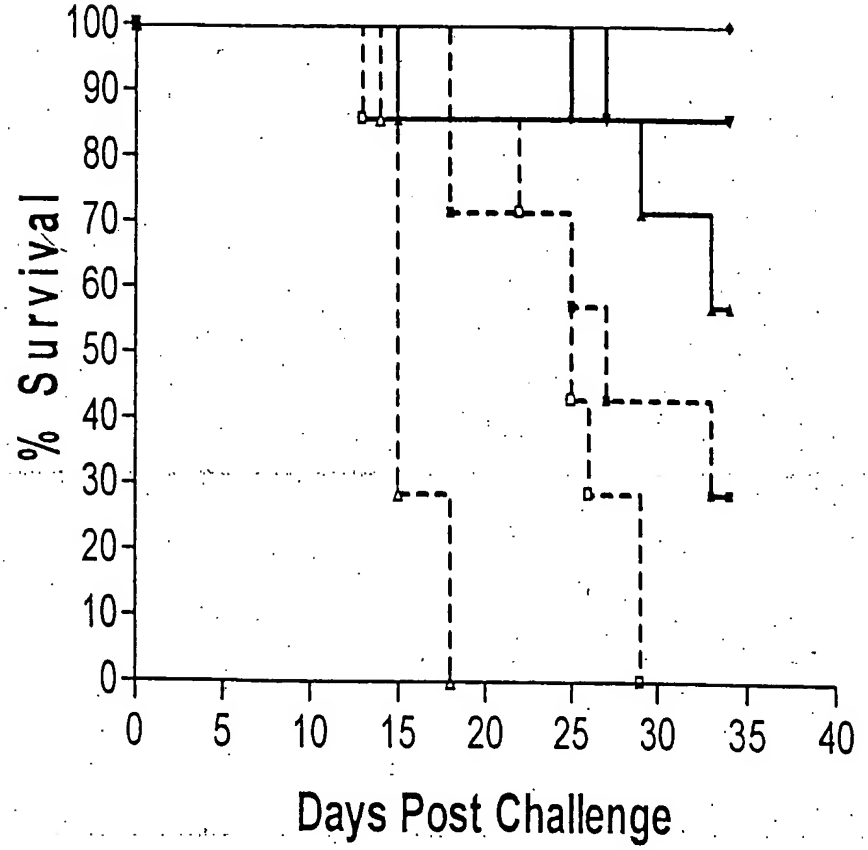
NEM 82

Evaluation of protective Ability of Mutant N. caninum



2B2[b]
2C3[a]
6H11[a]
6G7[a]
TS-8[a]
Normal cells[b]
No Cells[a, b]

[a] sig. diff. from normal

[b] sig. diff. from TS-8

EXHIBIT C

NEM 83

Evaluation of Protective Ability of Mutant N. caninum

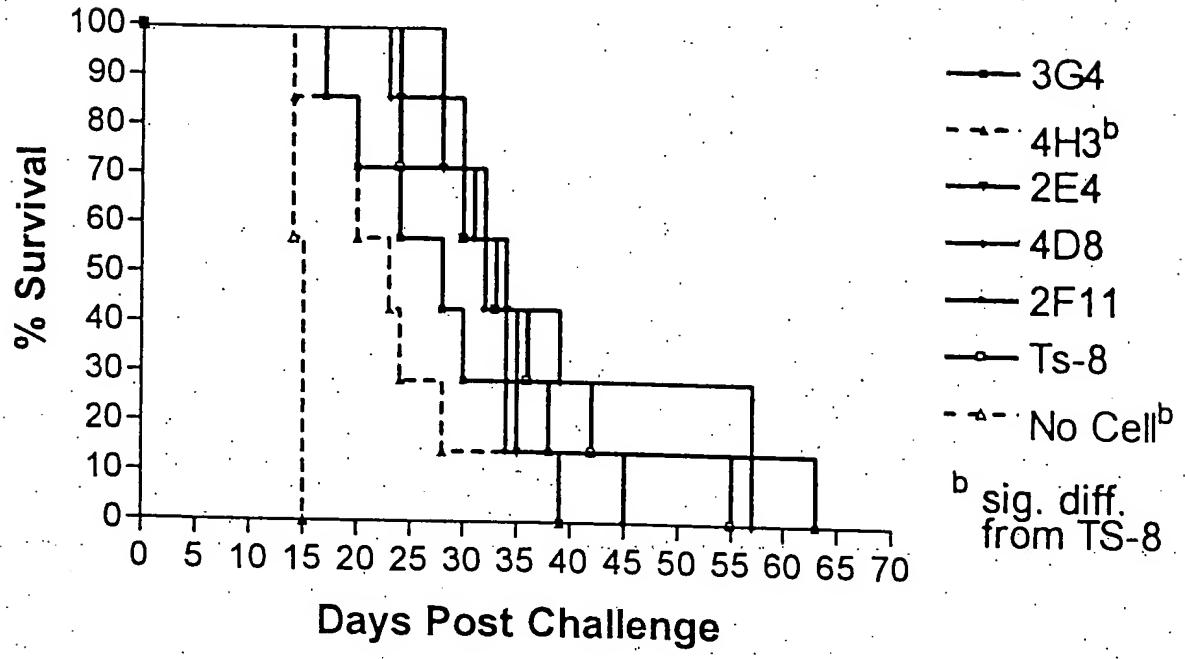
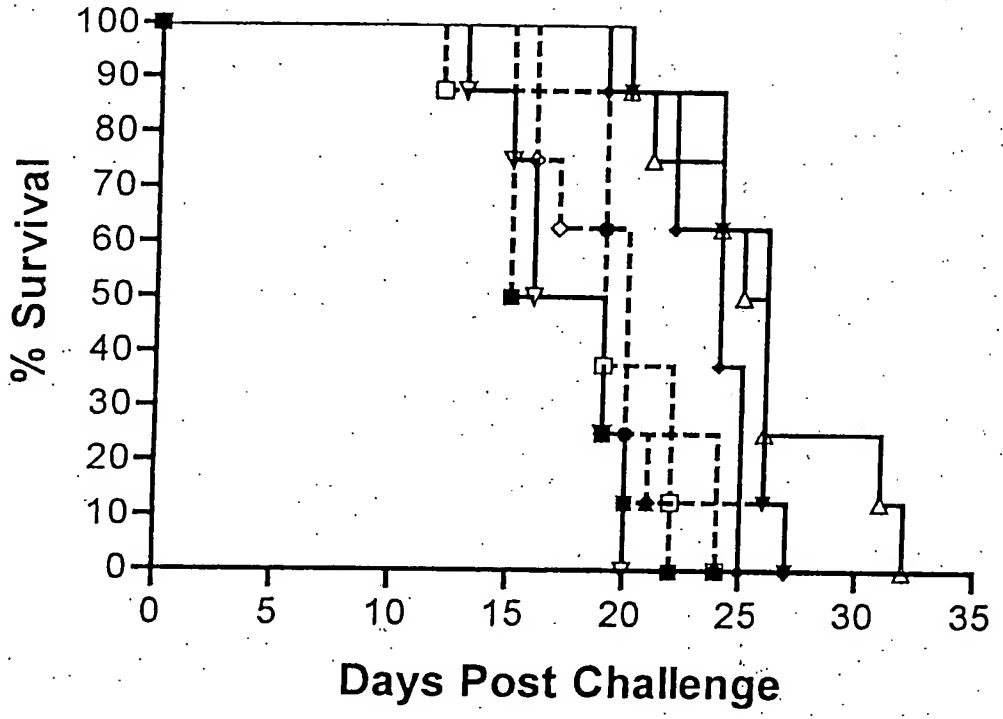


EXHIBIT D

NEM 92

Comparison of the Pathogenicity of N. caninum Mutants With Parental Strains in SCID/bg Mice



- 3G4
- 2F11
- 4D8[a]
- 6H11[b]
- 6G7
- 2E4
- TS-8[a]
- NC-1
- NC-2

[a]survival curves sig. diff. from parent at 0.05

[b]survival curves sig. diff. from parent at 0.09

EXHIBIT E

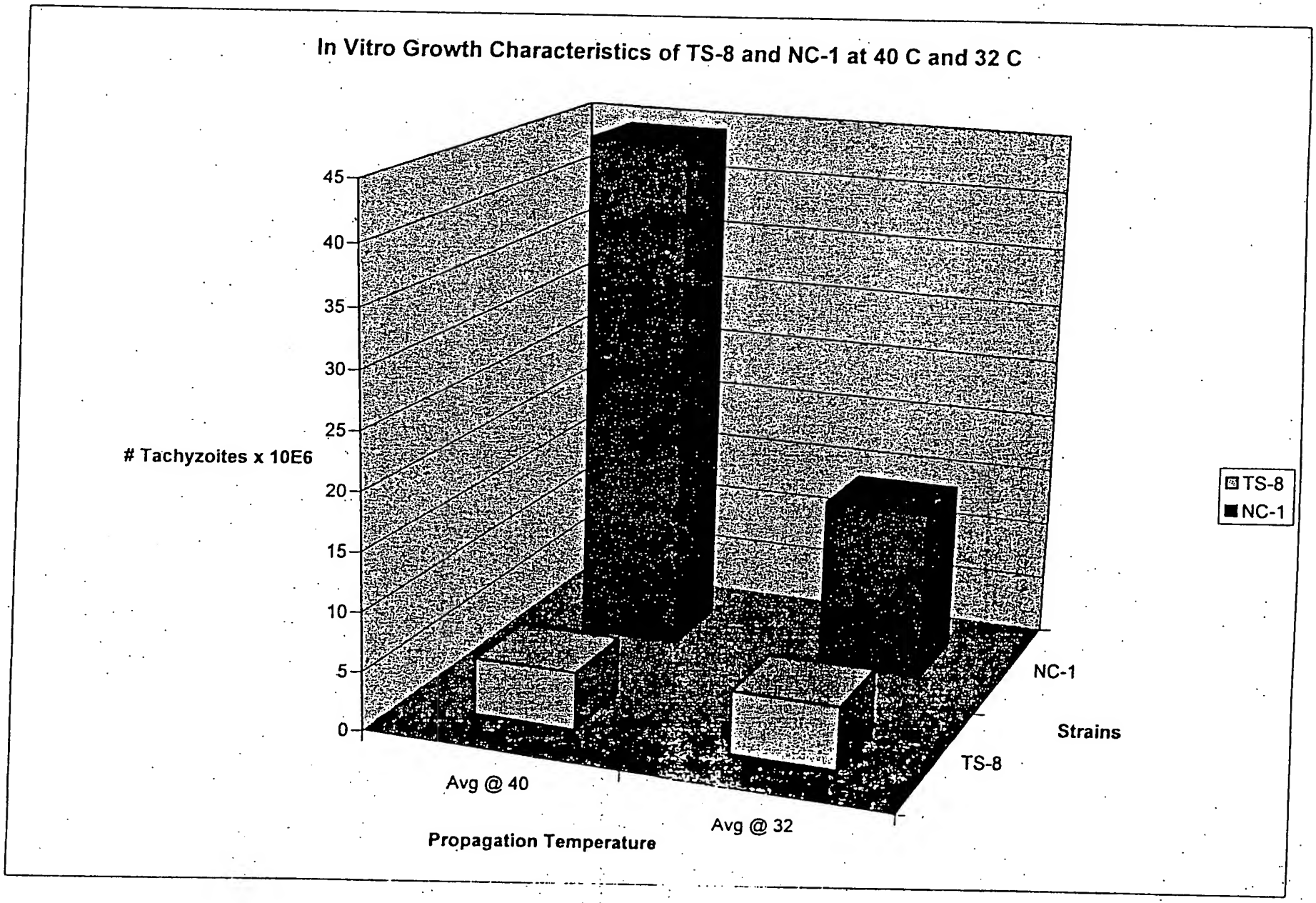

In Vitro Growth Characteristics of TS-8 and NC-1 at 40 C and 32 C
Tachyzoites x 10E6
45
40
35
30
25
20
15
10
5
0
Avg @ 40
Avg @ 32
Propagation Temperature
NC-1
TS-8
Strains
TS-8
NC-1

EXHIBIT F

In Vitro Growth Characteristics of N. caninum Mutants and NC-2 at 40C and 32C

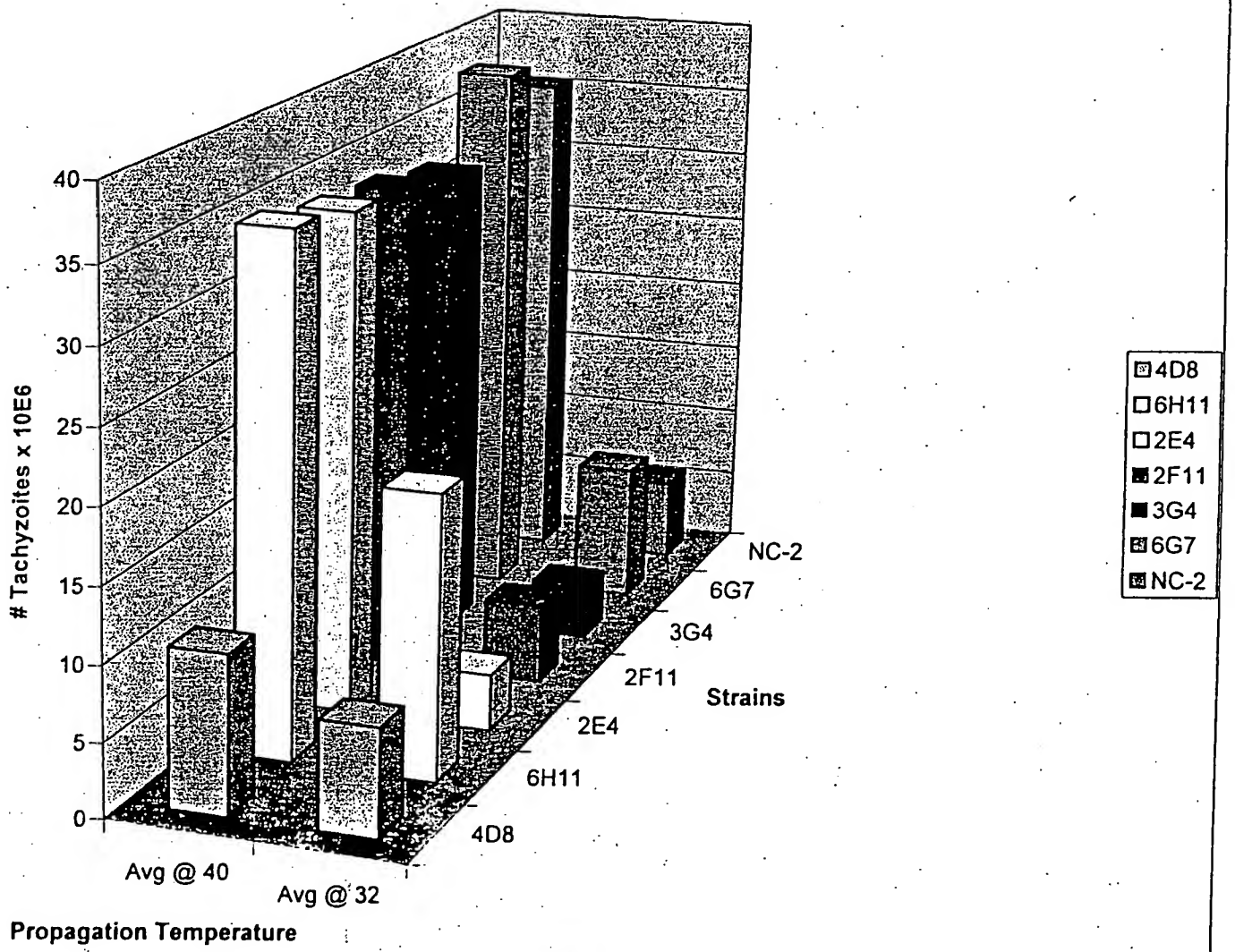


EXHIBIT G